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Sterol Composition of Isolates of *Erysiphe graminis* f.sp. *tritici* Differing in Sensitivity to Fenpropimorph

Antonius J. G. Engels & Maarten A. De Waard*

Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands

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Abstract: Isolates of *Erysiphe graminis* f.sp. *tritici* with wild-type or reduced sensitivity to fenpropimorph were similar in sterol composition, viz. ergosta-5, 24(28)-dienol (±90%) and episterol (±10%). Following treatment with fenpropimorph, the relative content of episterol increased in conidia of all isolates tested, while that of ergosta-5,24(28)-dienol decreased. These results suggest that fenpropimorph, under the test conditions used, does not inhibit activity of sterol Δ^{14}-reductase or $\Delta^8 \rightarrow \Delta^7$-isomerase but probably interferes with the final part of the demethyl sterol synthesis. However, modifications in this part of the pathway are probably not responsible for the decreased sensitivity of the pathogen to the fungicide. © 1998 SCI.

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1 INTRODUCTION

The obligate fungus *Erysiphe graminis* DC f.sp. *tritici* Marchal, the causal agent of wheat powdery mildew, is an important pathogen causing considerable yield reductions in wheat worldwide. For this reason, the chemical industry has intensively searched for compounds active against this fungus, and most cereal fungicides that have been developed are effective against powdery mildews. The most prominent group of systemic fungicides for mildew control are the sterol biosynthesis inhibitors (SBIs).

The morpholine fenpropimorph, a representative of the SBIs, has been used successfully in agriculture against powdery mildews of cereals, cereal rusts and leaf blotch of barley.[1] Morpholines are generally considered to have a dual mode of action. They inhibit both sterol Δ^{14}-reduction and Δ^8-Δ^7-isomerization to different degrees, depending on the structure of the inhibitor and the fungal species under investigation.[2] Cell-free studies with yeast reveal that tridemorph specifically inhibits the activity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase, while fenpropimorph and fenpropidin strongly inhibit both sterol Δ^{14}-reductase and sterol $\Delta^8 \rightarrow \Delta^7$-isomerase activity.[3]

One of the main risks of modern fungicides is resistance development in the target organisms. This risk of resistance development also applies to fenpropimorph in the control of *E. graminis* f.sp. *tritici*. A small decrease in the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph has been found in a number of western European countries.[4–7] To date, the biochemical mechanism responsible for reduced sensitivity to fenpropimorph in powdery mildews has not been investigated. Elucidation of this mechanism is important for designing anti-resistance strategies and for the development of fungicides active against fungicide-resistant populations.

Previous studies have revealed that the sterol biosynthetic pathway of *E. graminis* f.sp. *tritici* and f.sp. *hordei* is slightly different from that in other fungi.[8–10] Mildew fungi do not synthesize ergosterol but the related demethyl sterols ergosta-5,24(28)-dienol and its Δ^7 analogue, ergosta-7,24(28)-dienol (episterol).

* To whom correspondence should be addressed.
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The aim of this study is to investigate whether conidia from isolates of *E. graminis* f.sp. *tritici* with a reduced sensitivity to fenpropimorph differ in sterol composition from those of wild-type isolates. In addition, the sterol composition of conidia from these isolates, collected from fenpropimorph-treated wheat plants, was analysed.

2 MATERIALS AND METHODS

2.1 Plants and fungal isolates

Wheat seedlings of cultivar 'Okapi' were used. This cultivar carries no genes for resistance to powdery mildew. Plants were grown in a greenhouse at 18°C for eight days.

Two wild-type isolates of *E. graminis* f.sp. *tritici*, LH and 67 (FS), and two isolates with reduced sensitivity to fenpropimorph, 3a and 16c (FR) were used. They were isolated in the Netherlands in 1982 (LH), 1983 (67) and 1989 (3a and 16c) and have been maintained on 'Okapi' in a climate room at 10°C under fluorescent light (Philips TLMF 40W/35 RS; 7000 Lux) during 16 h a day and 80% RH. In foliar spray tests, the EC_{50} value of fenpropimorph for the FS-isolates LH and 67 is about 1·5 μg ml^{-1} and for the FR-isolates 3a and 16c about 12 and 13·5 μg ml^{-1}, respectively.

2.2 Preventive foliar spray test

Seedlings were sprayed with fenpropimorph prior to inoculation with *E. graminis* f.sp. *tritici*. Eight-day-old wheat seedlings (*c.* 25) in 0.2-litre pots (6 × 6 cm) were sprayed to run-off in a spray cabinet with 25 ml of an aqueous dispersion of a fenpropimorph 750 g litre^{-1} EC ('Corbel') for 2 min at a pressure of 0·8 bar. The concentrations used were 0, 1·5, 10 and 25 μg fenpropimorph ml^{-1}. After spraying, seedlings were grouped per concentration and 3 m apart in order to avoid cross-contamination from vapour-phase activity of fenpropimorph, and left to dry for 2 h.

Seedlings were inoculated with mildew by shaking infected seedlings, abundantly sporulating, above fenpropimorph-sprayed seedlings. In this way, the seedlings received a dense inoculum of powdery mildew conidia. FS-isolates were inoculated onto seedlings sprayed with 0 (control) and 1·5 μg fenpropimorph ml^{-1} (*c.* EC_{50} value) whereas FR-isolates were inoculated onto seedlings sprayed with 0, 10 (*c.* EC_{50} value) and 25 μg fenpropimorph ml^{-1}. For each isolate and concentration, 30 pots with seedlings were inoculated. Immediately after inoculation, seedlings were covered with a cellophane bag, grouped per concentration and incubated in a climate room at 18°C.

After 10 days of incubation, conidia of the fungus were harvested using a cyclone-type spore collector connected to a small vacuum pump. The conidia were freeze-dried and stored at −80°C in the dark. Control and fenpropimorph treatments were carried out twice and three times, respectively.

2.3 Curative foliar spray test

Seedlings were first inoculated and five days later sprayed with fenpropimorph. Seedlings inoculated with FS-isolates were sprayed with 0 and 1·5 and seedlings inoculated with FR-isolates with 0 and 10 μg fenpropimorph ml^{-1}, respectively. Six days after inoculation, conidia were collected and treated in the same way as described for the preventive foliar spray test. All treatments were carried out in duplicate.

2.4 Sterol extraction

Sterol extraction was performed by alkaline saponification.[11] Conidia (0·1 g dry weight) were acid-labilised by resuspending in hydrochloric acid (0·1 M; 2 ml), and heated at 100°C for 20 min in screw-capped tubes. Cholesterol was added as an internal standard. After heating, conidia were washed in distilled water and resuspended in potassium hydroxide in 60% methanol (4·3 M; 5 ml) and pyrogallol in methanol (4·0 mM; 2 ml). The mixture was refluxed for 1–2 h at 60–80°C.[12]

After cooling, the saponification mix was extracted by addition of heptane (3 ml). The phases were separated by centrifugation at 500*g* for 1 min. The top heptane layer was collected and the remaining solution was re-extracted twice. Any emulsions occurring were dispersed by adding methanol (0·5 ml). Anhydrous sodium sulfate was added to remove any water in the sample. The combined heptane extracts were evaporated to dryness under nitrogen at 40°C and sterol residues were stored at −20°C.

2.5 GC and GC–MS

Sterol residues were dissolved in a mixture of anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane (7 + 2 + 1, by volume) and left overnight at room temperature. The resulting trimethylsilyl (TMSi) sterol derivatives were analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). GC was performed on a Hewlett-Packard 5890 gas chromatograph, fitted with a flame ionization detector, using a DB-17 fused silica capillary column (30 m × 0·241 mm ID; film thickness 0·25 μm). Analyses were conducted isothermally at an oven temperature of 260°C and an injection temperature of 300°C. The carrier gas was hydrogen with a flow rate of 1 ml min^{-1}. Peak areas were determined using a data processor. GC–MS was performed on a

Hewlett-Packard 5870 series Mass Selective Detector (MSD) coupled to a Hewlett-Packard 5890 gas chromatograph. TMSi derivatives were separated on a BP-17 fused silica capillary column (30 m × 0·25 mm ID; film thickness 0·25 μm) at 260°C. The carrier gas was helium and the pressure at the column inlet was 3·2 bar. An on-column injector was utilized and the column was coupled directly to the ion source of the MSD. The ion source temperature was 200°C, electron energy 70 eV and electron current 100 μA. The cholesterol internal standard was used to calculate relative retention times (RR_t) and total amount of sterols. Identification was based on comparison with references to published data on mass spectra and relative retention times of known sterols.

2.6 Data analysis

The ratio between the Δ^7 and Δ^5 sterols was analysed using Analysis of Variance (ANOVA). Whenever significant differences were found ($P < 0{\cdot}05$), treatment means were separated using the Least Significant Difference (LSD) test.

3 RESULTS

3.1 Identification of sterols

Total sterol content was about 4 μg mg^{-1} dry weight of conidia. Both GC and GC–MS analysis revealed two peaks with RR_t values (cholesterol TMSi ether = 1·000) of 1·313 and 1·529, respectively. Mass spectra ($[M]^+$ (m/z)) and relative intensity (% of base peak) of the peak with RR_t 1·313 were 470 $[M]^+$ (31), 455 $[M\text{-}CH_3]^+$ (22), 387 (32), 386 $[M\text{-}C_6H_{12}]^+$ (100), 380 $[M\text{-}TMSiOH]^+$ (56), 371 (26), 365 $[M\text{-}CH_3\text{-}TMSiOH]^+$ (38), 344 $[M\text{-}(SC + H)]^+$ (23), 343 $[M\text{-}(SC + 2H)]^+$ (55), 342 (31), 341 $[M\text{-}TMSiOH = CHCH_2CH_3]^+$ (60), 340 (21), 296 (65), 281 (37), 257 (29), 255 $[M\text{-}TMSiOH\text{-}SC]^+$ (18), 253 $[M\text{-}TMSiOH\text{-}(SC + 2H)]^+$ (44), 213 $[M\text{-}TMSiOH\text{-}SC\text{-}42]^+$ (26) and those of the peak with RR_t 1·529 were 470 $[M]^+$ (8), 455 $[M\text{-}CH_3]^+$ (17), 386 $[M\text{-}C_6H_{12}]^+$ (34), 344 $[M\text{-}(SC + H)]^+$ (31), 343 $[M\text{-}(SC + 2H)]^+$ (100), 255 $[M\text{-}TMSiOH\text{-}SC]^+$ (19), 253 $[M\text{-}TMSiOH\text{-}(SC + 2H)]^+$ (13), 227 (18), 213 $[M\text{-}TMSiOH\text{-}SC\text{-}42]^+$ (21). These results and comparison with data from literature[9] identified the compounds with RR_t 1·313 and 1·529 as ergosta-5,24(28)-dienol and episterol, respectively. No differences in sterol composition were observed between isolates with wild-type or reduced sensitivity to fenpropimorph.

3.2 Sterol analysis of conidia from preventive foliar spray tests

Analysis of conidia from FS- and FR-isolates produced after a preventive treatment with fenpropimorph demonstrated that they had the same sterol composition as conidia present in control treatments, i.e. ergosta-5,24(28)-dienol (89–91%) and episterol (9–11%) (Table 1). However, after fenpropimorph treatment, the content of episterol increased and that of ergosta-5-24(28)-dienol decreased. As a result, the ratio between Δ^7 and Δ^5 sterols increased as well. This increase was statistically significant for the FS-isolate LH.

TABLE 1
Effect of Fenpropimorph on Sterol Composition of Conidia from Isolates of *Erysiphe graminis* f.sp. *tritici* differing in Sensitivity to Fenpropimorph in Preventive Foliar Spray Tests

Isolate[a]	*Fenpropimorph* ($\mu g\ ml^{-1}$)	*Ergosta-5,24(28)-dienol* (%)	*Episterol* (%)	*Ratio* $\Delta^7 : \Delta^5$ *sterols*[b]	*Sterol content* ($\mu g\ mg^{-1}$ *dry wt*)
LH	0	89·0	11·0	0·12 a	4·1
LH	1·5	83·2	16·8	0·20 b	3·8
67	0	88·3	11·7	0·13 a	3·8
67	1·5	85·4	14·6	0·17 a	3·9
3a	0	88·7	11·3	0·13 a	3·6
3a	10	86·9	13·1	0·15 a	4·3
3a	25	85·1	14·9	0·18 a	3·8
16c	0	88·0	12·0	0·14 a	4·2
16c	10	86·7	13·3	0·15 a	4·6
16c	25	85·6	14·4	0·17 a	5·0

[a] LH and 67: isolates with wild-type sensitivity to fenpropimorph; 3a and 16c: isolates with reduced sensitivity to fenpropimorph.
[b] Means followed by the same letter in the same column do not differ significantly, $P < 0{\cdot}05$.

3.3 Sterol analysis of conidia from curative foliar spray tests

After curative treatment with fenpropimorph, conidia from FS- and FR-isolates also contained the two sterols, ergosta-5,24(28)-dienol and episterol, already detected in conidia collected from isolates growing on untreated seedlings. The increase of episterol (%) after the curative treatment was obvious in all treatments (Table 2). As a result, the ratio between Δ^7 and Δ^5 sterols increased significantly in sterol extracts of all isolates tested.

4 DISCUSSION

Sterols from conidia of FS- and FR-isolates of *E. graminis* f.sp. *tritici* were identified as ergosta-5,24(28)-dienol and episterol and the proportions of the two sterols were *c.* 90% and *c.* 10%, respectively. Similar sterol compositions have been described for the same and related mildew pathogens, although in some species traces of other sterols were identified.[8] In the absence of fenpropimorph, FS- and FS-isolates displayed no difference in sterol composition. This indicates that an altered sterol composition cannot explain the reduced sensitivity to fenpropimorph in FR-isolates. Similar results were found for isolates of *E. graminis* f.sp. *hordei* and f.sp. *tritici* differing in sensitivity to DMIs.[10]

In three out of four isolates studied in the curative foliar spray test with fenpropimorph, the sterol content was almost halved. In both the preventive and curative treatment no accumulation of $\Delta^{8,14}$-sterols such as ergosta-8,14,24(28)-trienol and of Δ^8-sterols such as fecosterol was observed, indicating that Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase, described in literature as the target enzymes of morpholines,[2] were not affected under the test conditions used. However, the proportional content of episterol increased. This resulted in a significant increase in the ratio between Δ^7 and Δ^5 sterols in conidia of all isolates tested, especially in the curative foliar spray tests. An increase of episterol was unexpected since this sterol is an intermediate in the final stage of the biosynthesis pathway. It is not clear whether the increase in episterol reflects a direct effect on the enzyme involved in the introduction of the 5(6)-double bond or, less likely, the reduction of the 7(8)-double bond. Nevertheless, results suggest that in *E. graminis* f.sp. *tritici*, fenpropimorph has a target site that affects the conversion of episterol in ergosta-5,24(28)-dienol. Similar results have been reported for tridemorph and fenpropidin.[13] Treatment of *E. graminis* f.sp. *hordei* and f.sp. *tritici* with sterol 14α-demethylation inhibitors (DMIs) led to an increase of eburicol, obtusifoliol and also episterol.[10,14] These results suggest that DMIs inhibit sterol 14α-demethylation and, in addition, affect the conversion of episterol into ergosta-5,24(28)-dienol. This may suggest that morpholines and DMIs have a common target site in the final part of the sterol biosynthesis pathway. It is possible that this target site also plays a role in the toxic activity of SBIs against powdery mildews. This target site is probably not involved in the mechanism of resistance to fenpropimorph, since no substantial difference in sterol composition of conidia from fenpropimorph-treated FS- and FR-isolates were observed.

Fenpropimorph treatment did not completely inhibit sterol synthesis, since ergosta-5,24(28)-dienol remained the major sterol in all experiments. This may be explained by the quality of the conidia analysed. Probably, fungal biomass produced after the fungicide treat-

TABLE 2
Effect of Fenpropimorph on Sterol Composition of Conidia from Isolates of *Erysiphe graminis* f.sp. *tritici* differing in Sensitivity to Fenpropimorph in Curative Foliar Spray Tests

Isolate[a]	*Fenpropimorph* ($\mu g\ ml^{-1}$)	*Ergosta-5,24(28)-dienol* (%)	*Episterol* (%)	*Ratio* $\Delta^7 : \Delta^5$ *sterols*[b]	*Sterol content* ($\mu g\ mg^{-1}$ dry wt)
LH	0	91·4	8·6	0·09 a	4·0
LH	1·5	86·2	13·8	0·16 b	4·0
67	0	88·1	11·9	0·14 a	3·8
67	1·5	83·2	16·8	0·20 b	1·9
3a	0	90·2	9·8	0·11 a	3·8
3a	10	86·2	13·8	0·16 b	2·0
16c	0	87·9	12·1	0·14 a	4·2
16c	10	73·2	26·8	0·37 b	3·0

[a] LH and 67: isolates with wild-type sensitivity to fenpropimorph; 3a and 16c: isolates with reduced sensitivity to fenpropimorph.
[b] Means followed by the same letter in the same column do not differ significantly, $P < 0{\cdot}05$.

ment partly originated from conidia that had escaped toxic effects of fenpropimorph, tested at its EC_{50} value. This assumption also explains why the sterol composition of conidia is more strongly affected after curative than after preventive treatment. Applications of fenpropimorph at lethal concentrations might have caused a more pronounced effect on sterol composition but would have hampered recovery of fungal biomass.

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